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Murine Cytomegalovirus: Detection of Latent Infection by Nucleic Acid Hybridization Technique

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The technique of nucleic acid hybridization was used to detect the presence of murine cytomegalovirus (MCMV)-specific deoxyribonucleic acid (DNA) in cell cultures and salivary gland tissues. The presence of approximately 4.5 and 0.2 genome equivalents per cell of MCMV-specific DNA was identified in cultures of salivary (ISG_2) and prostate gland (IP) cells, respectively. These cells, derived from animals with experimentally induced latent infections, were negative for virus-specific antigens by immunofluorescence and on electron microscopy revealed no visible evidence of the presence of herpesviruses. A cell line derived from the salivary gland of an uninoculated animal (NSG_2) was also found to possess MCMV-specific DNA (0.2 genome equivalents per cell). For this reason, salivary gland tissues from uninoculated animals supplied as "specific pathogen-free" mice by three commercial sources were tested upon arrival for the presence of MCMV-specific DNA. MCMV-specific DNA was detectable in pooled salivary gland extracts from uninoculated animals derived from two commercial sources. All of these animals were seronegative and virus negative by conventional infectivity assays.

Although murine cytomegalovirus (MCMV) may establish latent infections in mice, the presence of latent virus in tissues often eludes detection. Initially after adult mice are inoculated with low doses of MCMV, virus can be found in the salivary gland in high titers and in other organs (spleen, kidneys, liver, lung, etc.) less frequently and in lower titers. After 3 months or more, virus may no longer be recoverable from tissue homogenates by conventional tissue culture assay. Latency has been established, since the presence of virus can be demonstrated by using explant and cocultivation techniques (2). Although tissue explant and cocultivation techniques provide means to activate latent MCMV, these techniques are not entirely reliable. In some cases, latently infected tissues can remain virus negative in an explant culture for a long period of time, and in still other instances, virus can never be activated by this method although additional evidence indicates that latent virus may nevertheless be present.

We previously reported the establishment of cell cultures derived from salivary and prostate glands of uninfected healthy mice as well as from mice with latent MCMV infection (2). Cell cultures that did not exhibit specific cytopathology had normal fibroblastic morphology, and, though superinfected with MCMV with the same efficiency as mouse embryonic fibroblast cultures, cells from infected and uninfected animals yielded different plaque morphologies (2).

We also demonstrated that latent MCMV could be reactivated in vivo by allogeneic blood transfusion (3). In one instance, however, MCMV was also recovered after isogenic transfusion, suggesting that latent virus might be present in some commercially supplied uninoculated mice.

In this paper, we report that nucleic acid hybridization techniques provide a sensitive and reliable method for the detection of latent MCMV. By employing these techniques, we have been able to demonstrate MCMV-specific genetic material in latently infected cell lines as well as in the salivary gland tissue of some commercially available mice supplied as "specific pathogen-free" animals.

MATERIALS AND METHODS

Virus. MCMV Smith strain was originally obtained from June Osborne of the University of Wisconsin and has been maintained in our animal facility by repeated passages in CD-1 mice. A 10% (wt/vol) homogenate of salivary gland was used as the virus stock.

Cell culture. Salivary gland cell lines NSG_2 (uninfected) and ISG_2 (latently infected) were established (tissue explants) from C_3H mice. Prostate gland cell lines NP_k (uninfected) and IP (latently infected) were established (tissue explants) from CD-1 mice. The procedures for the tissue explant culture, the estab-

lishment of cell lines from explants, and the maintenance of cell cultures have been previously reported (2).

A total of six groups were tested, each group consisting of 10 to 20 specific pathogen-free mice. Three groups were taken from our own animal care facilities. Three additional groups were obtained on separate dates directly from three different commercial sources. In the latter instances, to prevent the acquisition of MCMV infection in the laboratory area, these commercially supplied mice were sacrificed immediately upon arrival. The mice were exsanguinated, and the individual serum samples derived were frozen for subsequent testing. Salivary gland tissues were pooled from all mice in each of the six groups and frozen for processing at a later date.

Preparation of labeled DNA. MCMV was propagated in mouse embryonic fibroblasts and purified from the extracellular fluid of virus-infected cells as described previously in connection with studies of human cytomegalovirus (4). Viral deoxyribonucleic acid (DNA) was then prepared from the purified virions by pronase-sodium dodecyl sulfate digestion. The resulting mixture was subjected to sucrose sedimentation and further purified by two cycles of CsCl gradient sedimentation to equilibrium. Purified viral DNA was labeled in vitro by Kornberg's enzyme repair synthesis to a specific activity of 2.7×10^6 cpm/μg of DNA (4).

Cellular DNA was extracted from homogenized tissues or cultured cells by pronase-sodium dodecyl sulfate treatment, followed by phenol extraction procedures. The ribonucleic acid was removed by ribonuclease digestion (50 μg/ml for 1 h) and subsequent phenol extraction.

DNA-DNA reassociation kinetics. The DNA-DNA reassociation kinetics analysis and S1 enzyme differential digestion were carried out as described previously (5). Briefly, sonicated ^{3}H-labeled MCMV DNA (0.01 μg; 2.7×10^4 cpm; specific activity, 2.7×10^6 cpm/μg) and cell DNA (2 mg) were mixed and denatured in 0.01 M tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.4) and 0.0025 M ethylenediaminetetraacetic acid at 103°C for 15 min. The salt concentration was adjusted to 0.9 M NaCl after denaturation, and total volume was adjusted to 1 ml with water. Hybridization was conducted at 66°C in a water bath. At various time intervals, a sample was removed and rapidly chilled on ice. The percentage of renaturation was measured by S1 enzyme differential digestion. The S1 enzyme digestion was carried out at 41°C in 0.035 M acetate buffer containing 0.001 M zinc chloride and 0.3 M sodium chloride (pH 4.5). The S1 enzyme-resistant double-stranded DNA was precipitated by trichloroacetic acid and counted by a Packard scintillation counter. The S1 enzyme was prepared (Enzyme Development Corporation, New York, N.Y.) by procedures described by Vogt (9).

Analysis of the DNA reassociation data. The theoretical equation derived by Britten and Kohne (1) is used for describing the kinetics of DNA-DNA reassociation: $1/fss = 1 + K\,C_0t$, where *fss* is the fraction of the DNA sequences of the radioactive probe which remain single stranded at time *t*. *K* is the second-order constant, and C_0 is the initial concentration of DNA in the solution, expressed as moles of nucleotide per liter. The C_0t 50 is calculated when the fraction $1/fss$ is equal to 2.

Indirect immunofluorescent antibody technique. For the purpose of demonstrating specific serum antibody to MCMV, an indirect immunofluorescent technique was employed. Secondary mouse embryonic cells were grown in 250-ml plastic flasks. Near-confluent cultures were infected with MCMV 24 h after they were seeded. Three days after infection, when cytopathic effects were apparent in 90% of the cells, the cultures were trypsinized, and the cells were washed twice with phosphate-buffered saline. After appropriate dilution, the suspended cells were dispensed into shallow wells on slides (Bellco Biological Glassware, Vineland, N.J.). Uninfected cell suspensions were prepared in a similar manner. After air-drying, the slides were washed in phosphate-buffered saline, rinsed in distilled water, dried, and fixed with cold acetone. The acetone-fixed slides were kept frozen at −70°C until used.

Serum samples to be tested were diluted serially (twofold) and applied to wells containing infected and uninfected cells. After overnight incubation at 4°C in a humidified chamber, the slides were washed in phosphate-buffered saline, rinsed in distilled water, and dried. Fluorescein-conjugated goat anti-mouse immunoglobulin G or anti-mouse immunoglobulin M serum (Cappel Laboratories Inc., Cochranville, Pa.) was added. After a further incubation (45 min at 37°C), the slides were washed, rinsed, dried, and examined using a microscope adapted for viewing immunofluorescence.

Immunofluorescence techniques were also employed to investigate the presence of MCMV-specific antigens in latently infected cells. The salivary and prostate cell lines (see above) to be tested were seeded in the wells of test slides. To remove nonspecific antibodies, MCMV antiserum prepared in mice was absorbed before use with tissue culture cells derived from the same strain of mouse used to produce the virus. Fluorescein-conjugated goat anti-mouse immunoglobulin G was employed to demonstrate the presence of MCMV antigens. Normal mouse embryonic fibroblasts and MCMV-infected mouse fibroblasts were used as controls.

Electron microscopy. Cell pellets were fixed in glutaraldehyde for electron microscopy. Standard procedures were used to embed tissues in epoxy resin, as well as to section, stain, and examine these materials with a transmission electron microscope.

RESULTS

Studies in experimentally infected animals. (i) DNA-DNA reassociation kinetics. Both the salivary and prostate cell lines (ISG_2 and IP) derived from animals latently infected with MCMV showed the presence of viral genetic material at the level of 4.5 and 0.2 genomic equivalents per cell, respectively (Fig. 1). The reassociation of viral DNA in ISG_2 latently infected salivary gland cells reached a plateau at 70% of the control level. In addition, a small amount of virus-specific material was also found in the salivary gland cell line NSG_2 derived from

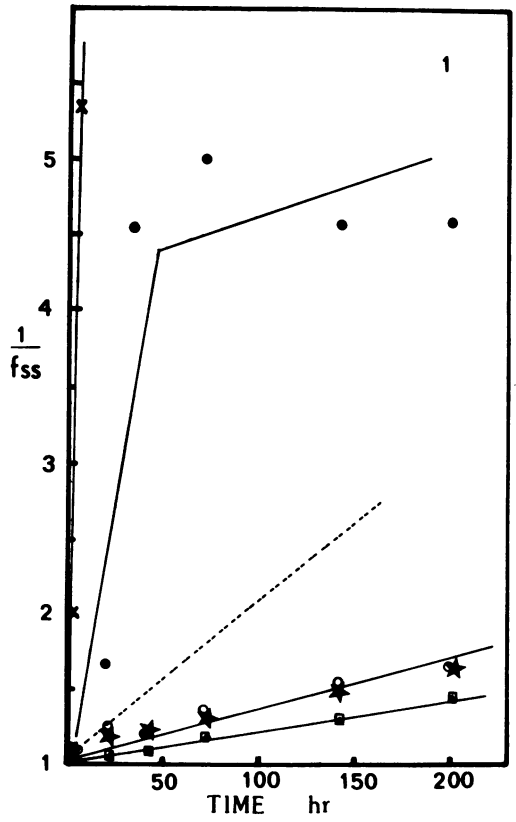

FIG. 1. *Reassociation kinetics analysis of tritiated MCMV DNA with DNA extracted from tissue culture-grown cells.* (○) *Uninfected salivary gland cells, NSG_2;* (●) *experimentally induced latently infected salivary gland cells, ISG_2;* (▲) *uninfected prostate gland cells, NP_k;* (★) *experimentally induced latently infected prostate gland cells, IP;* (□) *calf thymus DNA;* (×) *MCMV DNA. The dotted line indicates the Theoretical line for one genome equivalent per cell.*

an uninoculated animal (about 0.2 genomic equivalent).

(ii) Surface antigens. No MCMV-specific immunofluorescence was detectable on the surface of any of the latently infected cells that showed the presence of viral genetic material by the DNA-DNA reassociation kinetic technique.

(iii) Electron microscopy. Electron microscopic examination of thin sections of all of these cells revealed no visible evidence of herpesviruses, although C-type particles were often found in both the salivary gland and prostate gland cells.

Studies in tissues of uninoculated mice. Two pools each of salivary gland tissues derived from Swiss white and C_3H mice were positive for MCMV genomic material, although these tissues were negative by conventional assay. These positive pools included those derived directly from commercial sources as well as those originally from the same two commercial firms, now maintained in our own animal facilities. DNA-DNA reassociation kinetics indicated the presence of 3 and 0.9 genomic equivalents, respectively, in the salivary glands of these Swiss and C_3H mice, obtained either directly from suppliers or from our own facilities. CD-1 mice obtained from a third commercial source, as well as those originally from this supply house but now maintained in our own mouse colonies, were found to be virus negative by this technique, as well as by conventional assay (Fig. 2).

(i) Histology. Tissue sections of salivary glands, both positive and negative for the

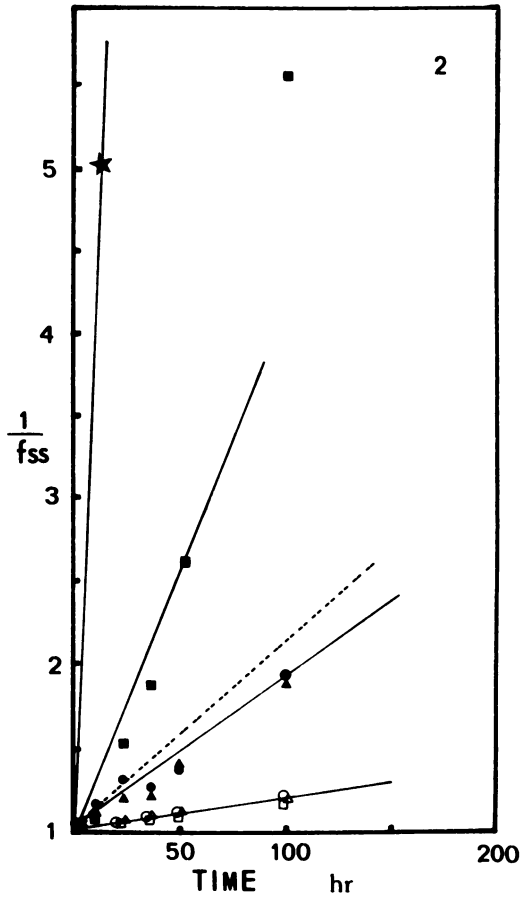

FIG. 2. *Reassociation kinetics analysis of tritiated MCMV DNA with DNA extracted from salivary gland tissue of mice from commercial and laboratory sources.* (■) *Swiss white mice;* (▲) *C_3H;* (△) *CD-1;* (○) *ALIF, CD-1 mice maintained in the Animal and Laboratory Isolation Facility at Duke University;* (●) *CD-1 with experimentally induced latent MCMV infection;* (□) *calf thymus DNA;* (★) *MCMV DNA. The dotted line indicates the theoretical line for one genome equivalent per cell.*

MCMV genome, were examined by light microscopy. Normal histology was observed in all samples obtained from uninoculated mice, and no inclusions were seen in these tissues. The tissue sections from mice with experimentally induced infections showed the presence of low-grade focal inflammation more than 1 month after virus could no longer be recovered from these tissues by conventional assay. Thereafter, the histology of the salivary gland gradually returned to normal, coincident with the establishment of latent infection. MCMV-specific DNA, however, could still be detected at a level as high as 0.5 genomic equivalents per cell even after viral latency was established and the histology had returned to normal.

(ii) Serum antibody to MCMV. The serum from individual animals in each group was tested for antibody to MCMV by complement fixation and by indirect immunofluorescence. None of the uninoculated mice, including those that were positive for this virus by the nucleic acid hybridization technique, possessed detectable antibody to MCMV. In contrast, all animals latently infected after inoculation of MCMV were antibody positive.

DISCUSSION

Latent MCMV infection can be reactivated by prolonged cocultivation, by explant techniques, by allogeneic transplantation, and by the use of immunosuppressive drugs (2, 6–8, 10). However, these methods for the demonstration of latent virus are time consuming, unpredictable, and of low efficiency.

Our studies indicate that the use of molecular hybridization techniques may reveal the presence of latent MCMV undetectable by conventional virological and serological methods. The molecular hybridization technique that we have employed identifies the presence of virus-specific genetic material, but does not distinguish between complete or portions of the genome. The fact that the reassociation of ISG_2 viral DNA reached a plateau (70% of control) may be due to recombinations between host and virus DNA or to the emergence of MCMV variants genetically slightly different from the virus used for the (DNA) probe.

These studies also indicate that mice commercially supplied as specific pathogen free may harbor latent MCMV. This finding probably explains the presence of a small amount of MCMV DNA (0.2 genome equivalent per cell) in a cell line (NSG_2) derived from an uninoculated C_3H mouse. The presence of MCMV DNA does not necessarily mean that this virus can be reactivated or will interfere with experimental procedures. However, investigators should be aware that these viral genes may be present in experimental animals. When the presence of latent MCMV in experimental hosts or cells is of concern, the technique of molecular hybridization provides a sensitive indicator for the detection of these viral genes.

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